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| 14. ABSTRACT We studied the expression of Alk and the effects of Alk mutations on learning and memory in mice. Concordant with studies in flies, we found enhanced retention of spatial memory in Alk mutant mice. Retention of spatial memory is a hippocampal dependent function. We also demonstrated expression of Alk throughout the adult murine hippocampus. The behavioral phenotype of Alk mutant mice is the opposite of the behavioral phenotype of Nf1 mutant mice. We hypothesize that the genetic interaction between Alk and Nf1 in mice is similar to the behavioral phenotypes of Alk and Nf1 mutations in flies and that pharmacologic or genetic inhibition of Alk in Nf1 mutant mice will attenuate or even rescue learning impairments in mice. This project involves a complicated breeding scheme. After fulfilling all the institutional guidelines and months of effort to generate mice for us, we only received 2 male NF1+/- mice on the 129 background on 10/09/2013 and 4 male and 3 female Alk KO mice on the C57BL/6J background on 9/25/2013. Therefore, we have put all our energy on generating breeder mice and experimental mice for this project. It is going extremely well; soon we will start the first experiment for this project. | | | | | |
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Introduction

Nf1 mutations, which occur in approximately 90% of patients with neurofibromatosis, are associated with cognitive impairment. Impaired academic performance is common and often requires special education^{1,2}. Mental retardation is seen in 6-7% of children, a percentage about three times higher than that in the general population. Specific learning disabilities in reading, spelling and math occur in 20% of children without overt central nervous system pathology³. Impairments in visual spatial processing and more complex executive functions have also been reported^{4,5}.

The mechanism underlying the cognitive impairments associated with neurofibromatosis is hard to study in humans. The phenotypes observed in mice indicate a specific function for Neurofibromin in the hippocampus⁶. The Morris water maze, an assay of murine hippocampus-dependent learning, has been employed to explore this phenotype⁶⁻⁸. In a specific test of the ability to recall the location of a non-visible, submerged platform based on remote visual cues, *Nf1* heterozygous mutant mice consistently show impaired spatial memory compared to wild-type controls. This phenotype is genetically and pharmacologically susceptible to modification by manipulation of Ras, a regulator of the MAP kinase (MAPK) signal transduction cascade⁶.

One of Neurofibromin's biochemical functions is to modulate signal transduction through the Ras-MAPK pathway. Neurofibromin is a negative regulator of this pathway that catalyzes the conversion of activated Ras-GTP to inactive ras-GDP^{7,9}. The Ras-MAPK signal transduction pathway is canonically responsive to receptor tyrosine kinase (RTK) activation. Recently, strong evidence has emerged from studies of *Drosophila* that activation of Anaplastic Lymphoma Kinase (Alk) by its ligand, Jelly belly (Jeb), is the physiologically relevant target of negative regulation by *Nf1*¹⁰. The link between Jeb/Alk signaling and Neurofibromin is supported by studies of *Nf1* regulation of body size and associative learning in flies. Based on the phenotypic resemblance between activation of Alk and inactivation of *Nf1* and genetic interactions between *Nf1* and *alk* in *Drosophila*, Gouzi et al. hypothesized that NF1 functions as a specific negative regulator of Alk signaling to enhance learning and memory. They showed that *Nf1* and *alk* mutations strongly interact with respect to both regulation of body size and olfactory associative learning. They also showed that the learning impairments in *Nf1* mutants could be corrected or rescued by genetic or pharmacologic inhibition of Alk. In support of the hypothesis that Neurofibromin acts directly downstream of Alk activation, they also demonstrated that expression of *Nf1* mRNA specifically in cells that also express *alk* was sufficient to rescue both body size and learning phenotypes of *Nf1* mutant flies¹⁰. This implies that Neurofibromin acts as a negative regulator of Alk activation and that inhibition of Alk is a potential therapeutic intervention to compensate for haplo-insufficiency of *Nf1*.

Alk was originally identified as a human proto-oncogene frequently activated by chromosomal translocation in lymphomas¹¹. It plays a causative role in a number of other human malignancies, including non-small cell lung cancer and neuroblastoma¹²⁻¹⁵. Orally active small molecule inhibitors have shown notable effectiveness in the treatment of lung cancer and are actively being tested for the treatment of neuroblastoma¹⁶⁻¹⁸.

The normal function of Alk in humans is less clear though its expression in both the developing and adult nervous system of mammals has focused recent investigations on behavioral phenotypes¹⁹. The hypothesis of behavioral and other neural functions of Alk in

humans is supported by a variety of studies in model organisms, including *Drosophila*, *C. elegans* and mice.

Behavioral functions for an Alk ligand were first described in *C. elegans*. An unbiased, forward genetic screen for mutations that effect integration of conflicting sensory inputs identified Hen-1, the *C. elegans* homologue of Jeb, as a signal that participated in behavioral response to simultaneous, conflicting attractive and aversive stimuli ²⁰. One of the most striking findings was that the Hen-1/Jeb requirement was not developmental. The behavioral phenotype could be rescued by a temporally controlled transgene only if it was expressed in adults. The behavioral phenotype of Hen-1/Jeb mutants included an effect on associative learning.

In *Drosophila*, Jeb and Alk were first characterized for their roles in early muscle development ²¹⁻²³. Investigation of their functions in the nervous system has required conditional genetic techniques to circumvent embryonic lethality. Several functions for Jeb and Alk have been established in the developing and adult *Drosophila* nervous system: 1) Jeb and Alk are essential for correct axon targeting of a subset of photoreceptors ²⁴; 2) Late embryonic maturation of the larval neuromuscular junction requires Jeb and Alk, though they are not required for axon targeting or synapse formation in this context ²⁵; 3) Jeb and Alk protect neurogenesis when developing larvae are nutritionally challenged ²⁶; and 4) Jeb and Alk have been implicated in associative learning and regulation of body size upstream of Nf1. Recently, Jeb activation of Alk in the larval neuromuscular junction, a glutamatergic synapse, was shown to be a potent negative regulator of synaptic transmission ²⁷

Body

We have studied the effects of Alk mutations on learning and memory in mice. Concordant with Gouzi et al's findings in flies, we find enhanced performance in retention of spatial memory in Alk mutant mice ²⁸. The behavioral phenotype of *Alk* mutant mice is the opposite of the behavioral phenotype found in *Nf1* mutant mice. Based on these data, we hypothesize that the genetic interaction between *Alk* and *Nf1* in mice is similar to the behavioral phenotypes of *alk* and *Nf1* mutations in flies. We further propose that pharmacologic and genetic inhibition of Alk in *Nf1* mutant mice will attenuate or even rescue learning defects in mice, as it does in flies.

The specific aim of our proposal is to test the effects of genetic and pharmacologic inhibition of Alk on retention of spatial memory in heterozygous *Nf1* mutant mice. If pharmacologic inhibition of Alk in mice rescues the learning defect of *Nf1* mutations this will provide the basis for pursuit of similar strategies in humans. We propose two approaches to test the strategy of inhibiting Alk to treat the cognitive impairment caused by heterozygous *Nf1* mutation in mice, one genetic the other pharmacologic. These two approaches are complimentary and will address potential weaknesses inherent to each separate approach.

Key Research Accomplishments

This project involves complicated breeding and two mutant mouse models that needed to be imported, after processing all the required paper work for it. Also, as the mice we requested

needed to be bred and become available first, this delayed the shipment of these mice. As part of Raber import 15197, we received 4 male and 3 female Alk KO mice on the C57BL/6J background on 9/25/2013 from Dr. Liliana Attisano at the University of Toronto. As part of Raber import 15330, we received 2 male NF1+/- mice, on the 129 genetic background, on 10/09/2013 from Dr. Nancy Ratner at the Cincinnati Children's Research Foundation. We started the breeding on 10/15/2013 (Breeding Part A) and the subsequent breeding part (B) on 3/18/14. The details and the number of pups generated so far is described below.

BREEDING PART A: Mated 10/15/13 (2 breeder boxes)

NF1-/+ , +/+

X = NF1-/+ or +/+; Alk-/+ ... 42 MICE: 21 F, 21 M; 10 NF1-/+ ,
ALK-/+ (4 F, 6 M) +/+ , Alk-/Alk- (KO)
+ 9 PUPS

BREEDING PART B: Started mating 3/18/14 (2 breeder boxes so far)

(NF1-/+ , ALK-/+) X 2 = NF1-/+ or +/+; Alk-/Alk- or Alk-/+ or +/+ (6 TOTAL GENOTYPES)
... 46 PUPS (so far)

In the first round of breeding, it is only necessary to determine the NF1 genotype, as all offspring are Alk-/+ (heterozygous). We determine which mice carry the mutant NF1 and ALK alleles by extracting DNA from a small portion of the animal's tail and subsequently performing polymerase chain reaction (PCR). Our desired portion of DNA is amplified through PCR and visualized through gel electrophoresis (example shown below in Fig. 1). The 400 base pair bands for Samples A and B show that the animal possesses a mutant allele of the NF1 gene. Sample C is homozygous for the wild type NF1 allele because a 200 base pair band is clearly visible on the gel. ALK and NF1 PCR primer sequences and protocols were obtained from the Attisano and Ratner laboratories, respectively.

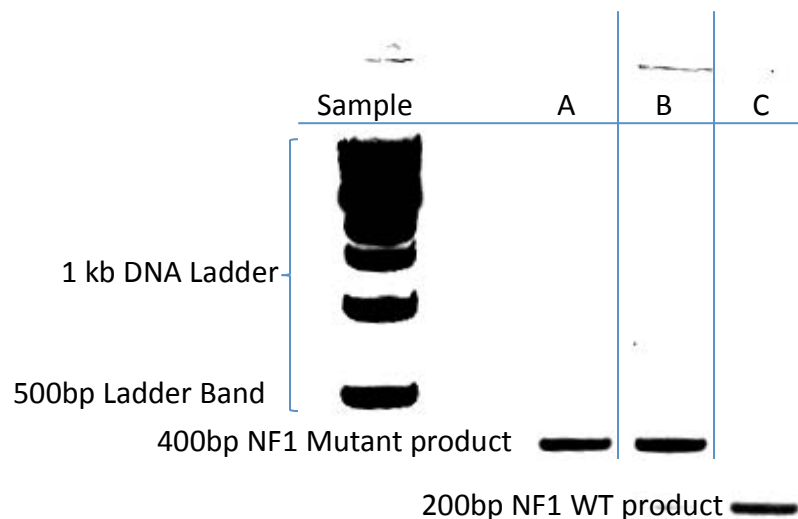


Fig. 1. Example of genotype determination by PCR. For details, see main text.

Reportable Outcomes

At this point, we have 46 mice available for testing. The first group of 3-month-old mice will be available in a few weeks and we are looking forward to the upcoming exciting experiments. More mice will become available as we continue this breeding effort.

Conclusions

In Yr 1 of this project, most effort was put into receiving the mouse models required for this project, develop the breeding efforts, and getting all regulatory requirements taken care of. The breeding effort is going well and we anticipate that we will not need to request additional mice for breeding, although we received way less mice than we would have used if we were able to purchase the mice or obtain more mice for breeding. As some of the genotypes are required for the pharmacological Aim as well, we anticipate that although receiving the mice took longer than anticipated that we will be able to quickly move from the genetic experiments to the pharmacological ones and finish all experiments within the second funding year of the project.

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